Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXII | *SEPTEMBER, 1943* | NUMBER 9 CONSECUTIVE No. 17

The Utility of Determination of Numbers and Dimensions of Glandular Scales in *Mentha* Species. I*

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In many aromatic plants such as members of *Labiatae* the aromatic principle, a volatile oil, is presumed to be formed in and stored by the glandular trichomes. Although there can be no doubt of this as a fact, yet no one up to the present apparently has proved with certainty that a portion of the essential oil in plants with external oil glands does not exist outside of the glands. This is one of the points of disagreement between the school headed by Charabot and that of Tschirch, Tunmann and others.

Charabot and his co-workers contended that the essential oil is manufactured in the chlorenchyma of the leaf, stem, etc., passing freely from cell to cell in the plant as needed but accumulating in greater proportions in the epidermis and particularly in the oil glands. On the other hand, Tschirch and Tunmann, as well as many other workers who have at the same time often disagreed with the resinogenous layer theory of these two, have maintained that the volatile oil is synthesized *in toto* within the glandular structure and once isolated remains there.[1]

Likewise, there has been an important difference in opinion by these 2 groups over whether the factors determining the quantity and quality of the volatile oil are: (*a*) external to the plant (such as temperature, insolation, humidity, etc.); or (*b*) internal factors, such as would exist if the oil entered into the metabolic processes of the plant. The first view is held by followers of Tschirch and Tunmann, the second by those of Charabot.

In the present investigation, it was assumed that the entirety of volatile oil is manufactured within the glandular scales and that external factors predominate in determining the characteristics of the volatile oil.

The objectives of the research here described were to determine what relation if

* This paper is an abstract of one portion of a dissertation presented to the Graduate Council of the University of Florida in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Presented before the Scientific Section of the A. PH. A., August 18, 1942.

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[1] Many references to this work appear in the body of the dissertation. There are too many to include in this paper. One outstanding contribution should be mentioned however, that by Tunmann (2).

any exists in certain *Mentha* species between numbers and dimensions of glandular scales on the one hand and oil-richness of the herb on the other. Hoelscher and Bacon (1) said a direct relationship exists between types and numbers of glandular hairs and the oil yield of peppermint.

EXPERIMENTAL

Before counts and measurements of the glandular structures on mint leaves could be made with accuracy, it was necessary to develop proper methods of study.

ENUMERATION OF GLANDULAR SCALES

Areas used.—Counts were carried out in 3 ways: Low power field counts were made on a fairly large number of fields taken at random in the general area of the leaf reported (*e. g.*, near apex). Where comparative studies were made of various leaves, counts were uniformly made in a region about halfway between apex and base and about halfway between margin and midrib. Average counts were then restated on the basis of 1 sq. mm.

Strips of standard length (8.3 mm.) and breadth (1.099 mm.) were counted on leaves as nearly as possible of an arbitrarily selected standard length (39 mm.). For this type of count, a special slide was used which had 11 transverse parallel rulings enclosing 10 rectangular areas of equal and uniform breadth. This slide was placed ruled side down over the leaf mounted lengthwise on a plain microslide. The 2 slides were then bound together by means of adhesive tape strips at each end. The rulings on the special slide were thus discernible when the low power objective was focused on the leaf surface. The length of the strip, like the leaf length, was selected arbitrarily.

Total counts for entire leaf surface were found practical only when the glandular scales were not numerous. Such counts are out of the question as routine procedure on typical mature leaves of a good quality of peppermint herb. Thus, one such leaf showed a total count (for both surfaces) of 16,600 glandular trichomes. Counting this number occupied 2 or 3 days of tedious observation. Another disadvantage is the difficulty of scrutinizing all parts of the leaf without any duplication. This is best accomplished by mounting the leaf securely on a slide set firmly in a mechanical stage, then inspecting parallel strips across the leaf from its apex to base, using the necessary precautions in moving the slide during the observations.

The estimation of total counts by multiplying representative unit area counts by the leaf area stated in terms of such units was found to be too inaccurate and too difficult to be of practical value. The method is inaccurate because area counts of glands were found to vary considerably at various points on the leaf and the method is difficult because of the problems encountered in determining leaf area accurately. Rather complex mathematical relations are involved on account of the variability in shape and size of individual leaves.

Attempts were made to project the image of the cleared leaf by means of (*a*) the regular lantern projector and (*b*) the microprojector. Both methods were found to be impractical, as was also the projection of the isolated glandular hairs mounted on the adhesive transparent cellulose tape with which they had been stripped from the leaf.

Preparation of Leaf Material.—The following techniques were tested:

Clearing of the entire leaf thickness by means of a clearing liquid. Water and aqueous solutions of salts proved poor for this. Clove oil, xylol, 95% alcohol, cresol and 20% (*v/v*) nitric acid were only fair. The best materials were 250% aqueous chloral hydrate solution (especially when heated just to boiling) and 5–10% aqueous sodium hydroxide solution. Tests showed that counts in these 2 reagents were ordinarily quite accurate, although heated chloral preparation sometimes contained torn, deformed, or even detached glandular scales.

Bleaching of entire leaf thickness (with chlorinated soda solution or potassium chlorate-chloral hydrate-nitric acid combination) resulted in only slightly more differentiation of glandular scales than is present in unbleached material, and there was also a certain amount of destruction or distortion of the glandular scales and of the tissue as a whole.

Staining with tincture of alkanna (fresh), Sudan III, safranin, methylene blue (0.5% in 95% alcohol) and methyl green (among others) failed to differentiate the glandular scales. Stains that dyed the scale cuticle also colored the extra-glandular cuticle to about the same intensity.

Stripping epidermis from the fresh leaf of *Mentha* species was attempted, following several types of pretreatment: (*a*) leaves left to wilt (for 42 hr.); (*b*) leaves left standing in water (for 2 days, for 17 days); (*c*) leaves left in refrigerator for several days, then boiled in water (for ½ min., for 10 min. and for 60 min.); (*d*) leaves heated just to boiling in 20% (*v/v*) nitric acid; (*e*) leaves laid in equal parts of alcohol and water with 5–10% stronger ammonia water (for 24 hr.).

None of these schemes for facilitating stripping of the epidermis proved superior to the method of incising the leaf surface with a razor blade and then gently raising with fine tweezers. By all these means only relatively small pieces of epidermis could be removed from the leaf.

The casting or impression method involves the application of a colored collodion solution or other plastic material to the leaf surface, letting it dry and finally stripping it off with care. This method has been studied by several investigators. It is of fair value for mint species, but rather distinctly inferior to the method next discussed.

The adhesive cellulose (cellophane) tape method is original with the present investigators, and it was

found simple and effective. The tape is laid evenly, with adhesive surface next to that of the leaf under study, and then it is carefully pressed down uniformly by means of any straight edge (*e. g.*, back of scalpel or ruler). The tape is then cautiously peeled off with tweezers (care being taken throughout not to touch the tape with fingers, etc.) and laid, adhesive side down, on a plain microslide. Examination may be made from either side or both sides. The method applies best to fresh material but with care it also proved of value for dried.

Polarized light causes the granular and crystalline material in the glandular scales to refract polarized light strongly. This proved of no practical importance for counts, however, since the same brilliant refraction did not appear after the necessary step of clearing the leaf (with chloral, etc.).

Untreated leaf material viewed by reflected light was found satisfactory for glandular scale counts with leaves both in the fresh state and in the flatly pressed-out dried material, when such leaves were not too thickly beset with mechanical or covering hairs, as was the case for instance with *Mentha longifolia* (L.) Hudson. Dried material, unless very old, was found preferable to use in making rapid counts, inasmuch as the glandular scales in this material shone forth brightly, whereas in the fresh leaf other portions of the epidermis reflected light, which was often found confusing.

Effect of Various Treatments of Leaf upon Glandular Scale Count.—The relative permanence of glandular scales on a leaf surface subjected to various treatments was studied.

Friction when applied with pressure was quite effective in removing glandular scales. Procedures tried included manipulation with the point of a needle (while examining under the low power of the microscope), vibration of the dried leaves in a container with a quantity of sand, marbles, mercury, etc., in a shaking machine and others. These treatments must be considered, however, more vigorous than those to which the leaf is submitted in the field, preceding the distillation process, or in the herbarium press.

The light friction from one leaf rubbing over another was found to have only a negligible effect on glandular scale counts. A box of marked dried leaves was placed in a shaking device. After 30 min. of violent movement, counts showed no appreciable change in the number of glandular scales. This was intended to approximately simulate conditions of collection, drying and handling mint outside and also the processes involved in preparing the herbarium sheet.

Drying with pressure: These factors in association are involved in the preparation of plants for the herbarium; the drying factor alone chiefly is concerned in commercial drying of the herb before distillation. These findings are of particular interest since the experimental conditions were considerably more critical than those in practice.

Blotting paper was placed over the leaves resting on microslides, and a definite pressure was well distributed on them from above by means of weights resting on a small board placed over the blotting paper. A pressure equivalent to 0.51 Gm./sq. mm. was applied in this way. In another experiment, a mercury column exercised a pressure of 0.63 Gm./sq. mm., which is equivalent to a pressure on the individual glandular scale of peppermint (0.005 sq. mm.) of about 3.15 mg. Careful microexamination showed that with the last-named pressure, a few of the glandular scales had been slightly distorted but not broken and the count remained the same.

Relation of Glandular Scale Count to Condition and Section of Bearing Organ.—In *Mentha*, glandular scales occur not only on both sides of the lamina of the foliage leaf, but also on the petiole and stem and on the floral parts (including the peduncle, pedicel, floral bracts, calyx, corolla, and anther). However, in this work counts were made only with foliage material.

Leaf size and age: In leaves of the same plant, the younger smaller leaves have a higher per area count of glandular scales than the older larger leaves, due to the fact for one thing that the glandular scales appear early, are relatively long-lived, and few new ones (if any) are formed in the mature plant; and for another thing in consequence of the expansion of the leaf lamina as the leaf matures.[2]

State of freshness or dryness of leaf: Peppermint leaf on drying shows an especially strong shrinkage, as has been noted by someone (3). Consequently, it is impossible to make comparative counts of glandular scales on fresh and on dried leaves.

Upper and lower surfaces of the leaf: The lower or dorsal leaf surface has shown in the species of *Mentha* examined constantly higher per area and total glandular scale counts than the upper or ventral surface. These differences are least in young small leaves, greatest in large matured leaves. Thus, in peppermint, counts varied from the low ratio (lower:upper) of approximately 2 (in small poorly leaves) to as high as 8.49 (in large well-developed leaves).[3]

Part of leaf in which counts are made: In general, those parts of the leaf which are oldest bear a relatively smaller number of trichomes because these parts have most nearly completed their expansion, and also because they have been exposed longer to influences which would cause the shedding

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[2] Although this explanation is quite obvious, it was overlooked by Tschirch and Tunmann when they proposed in several papers (4, 5, 6) that young leaves should be used in preference to older as a source of volatile oil. (*cf.* (7)) Tschirch, however rather belatedly admitted this factuality (8).

[3] This appears to result partly from the inherent tendency to develop more trichomes on the lower surface while in the bud but partly also to the protection afforded by this position, the lower part of the leaf being less subject to the action of atmospheric effects, animal organisms, etc. There are also generally more protective covering trichomes on the lower surface.

of the scales. Thus, the apex of the leaf has a lower per area count than the base. This difference is ordinarily greater on the lower than on the upper side of the leaf. Some typical averages are indicated in Table I.

TABLE I.—NUMBER OF GLANDULAR SCALES PER SQUARE MILLIMETER AT VARIOUS REGIONS OF LEAF IN PEPPERMINT

Region	Leaf A		Leaf B	
	Lower	Upper	Lower	Upper
Near apex	4.7	1.7	13.7	3.3
Near base	14.9	2.8	16.9	2.8
Near middle lateral margin	11.8	3.1	16.7	2.9
Near center of mid-rib	14.5	1.6	16.1	...

Environmental conditions of growth: Although sun leaves were found to have a distinctly higher per area count of glandular scales than shade leaves growing on the same plant, this difference undoubtedly depends in large part or altogether on the larger size of the shade leaves in the particular cases studied.

Plants growing in the winter season (in greenhouse) and plants in highly deficient nutrient media (as in sterile sand watered with distilled water or in unsuitable nutrient solutions) showed marked deficiency in numbers of glandular scales, as compared to plants growing under better conditions, as outside during the summer in properly treated soils.

Difference in plants growing at different latitudes: Indiana stock of commercial peppermint[4] was grown in Florida in the greenhouse.[5] An indication of the results is found in the data from a few averages of counts recorded in Table II, which also includes data for peppermint stock which has been growing for several years in the Medicinal Plant Garden of the University of Florida.

Relationship between glandular scale counts and richness of leaf in volatile oil: A series of specimens of leaves and tops of commercial peppermint plants from Indiana fields of known per acre yield was obtained in carefully collected and pressed condition.[6] Results of study of this material are reported in part in Table III.

TABLE III.—GLANDULAR SCALE COUNTS AND PEPPERMINT OIL YIELDS OF HERB

Where Collected in Indiana	Date When Collected	Acre Yield, Lb.	Sq. Mm. Counts[a]	Standard Strip Counts[a]
Winamac	Aug. 4, 1941	20	15.4	154
Winamac	Aug. 6, 1941	35	17.7	183
Winamac	Aug. 4, 1941	45	12.8	93
Walkerton	Aug. 14, 1941	50	25.6	239
Walkerton	Aug. 14, 1941	52	19.0	121
Walkerton	Aug. 14, 1941	55	17.7	180
Walkerton	Aug. 14, 1941	57	16.3	200

[a] All counts in this table were of lower leaf surfaces.

TABLE II.—GLANDULAR SCALE COUNTS IN FLORIDA- AND INDIANA-GROWN PEPPERMINT LEAVES[a]

Where Plants Were Grown	Time of Collection of Leaves	Number Glands per Sq. Mm. Upper	Lower
Indiana			
Near Walkerton	Aug. 14, 1941	3.0	19.7
Near Winamac	Aug. 4, 1941	2.15	15.3
Near Lafayette	Aug. 14, 1941	6.2	18.9
Near Winamac[b]	Aug. 4, 1941[b]	2.9[b]	10.2[b]
Gainesville, Florida			
Medicinal Plant Garden	Apr. 8, 1941	1.8	4.5
Medicinal Plant Garden	May 30, 1941	5.4	14.4
Medicinal Plant Garden	July 19, 1941	..	15.4
Medicinal Plant Garden[c]	Apr. 8, 1941[c]	1.3[c]	4.3[c]
Medicinal Plant Garden[d]	Apr. 8, 1941[d]	..[d]	2.0[d]
Greenhouse			
Garden stock[e]	Dec. 31, 1940	0.7	0.9
Garden stock[e]	Jan. 31, 1941	0.6	1.2
Garden stock[e]	Apr. 26, 1941	1.4	1.9
Indiana stock[f]	Nov. 16, 1941	..	4.8
Indiana stock[f,b]	Nov. 16, 1941[b]	..	5.7[b]

[a] All counts were made near leaf center to one side of mid-rib on leaves of as uniform size as possible.
[b] American peppermint. All others are English peppermint.
[c] Shaded plants; those preceding grew in open sunlight.
[d] Growing in deep shade.
[e] Sand cultures receiving a standard nutrient solution.
[f] Growing in a rich soil with adequate moisture and well drained.

From this compilation, in which entries have been arranged in the order of increasing oil yield per acre, it appears that only an approximate idea may be gained of the relative richness in oil in the herb from gland counts made on selected leaves; or at least from the relatively limited number utilized.

[4] Kindly supplied by Dr. R. C. Baines, Department of Botany, Purdue University, Lafayette, Ind.

[5] The stock was received too late for outside cultivation, the results of which would have been most interesting as to gland counts.

[6] Furnished through the kindness of Dr. R. C. Baines, Department of Botany, Purdue University, Lafayette, Ind.

Thus, leaves from a good production area sometimes gave lower gland counts than leaves of a poorer area.

MEASUREMENTS OF GLANDULAR SCALES

As with the making of counts, so likewise in determining the dimensions of glandular scales, it was first found necessary to determine a proper technique.

Glandular scales were measured on the fresh, dried and liquid-preserved leaves, respectively, both *in situ* and when separated from the leaf. Most measurements were made of lateral dimension (tangential to leaf surface) since the profile vertical to the leaf surface is less distinct and hence the vertical diameter less accurately measurable.

Effect of Liquid Mountants.—The glands are most readily and accurately measured when immersed in a liquid; in air mounts, it was often difficult to define them clearly. Hence, tests were made to find the effect of liquid treatment on scale dimensions.

(*a*) Chloral hydrate (250% aqueous solution) in the cold caused a steady increase in lateral diameter up to in excess of 30% and, after a period of minutes, to the point of bursting usually. Boiling in chloral solution of this strength uniformly ruptured the gland at once.

(*b*) Ethyl alcohol (95%) also caused swelling, although to a lesser extent than the preceding.

(*c*) Distilled water showed small and rather indeterminate effects on diametric measurements.

(*d*) Formalin-acetic acid solution[7] acted to decrease lateral diameters of glands in fresh material but increased such diameters in dried herbarium material (11.3% in 5 hr.).

Condition and Part of Plant.—Age and size of leaf: Very young or very small leaves appeared to have slightly smaller glandular scales. Otherwise, there appeared to be no correlation between size of leaf and size of glandular scale.

Fresh and dried leaf: A very slight decrease in lateral diameter (*e. g.*, 3.3% in one series of *Mentha citrata* after 21 days of drying) was observable on desiccating the leaf.

Part of plant: Glandular scales from various parts of the plant varied considerably in size. Thus, lateral diameters of glands on calyx and corolla were considerably larger (typically about 25%) than those of leaf glands.

Part of lamina: Glandular scales on larger veins were consistently larger than those not, a difference which amounted to as much as 20%.

Surface of lamina: Glandular scales on the upper (ventral) surface were found quite uniformly larger than those on the lower (dorsal) surface. This may be an indication that sunlight some how stimulates growth of the glandular scale.[8]

Sun leaf and shade leaf: Glandular scales are definitely larger on plants in the open than those shaded. This appears again to confirm the view that sunlight stimulates glandular scale growth in some manner.

DISCUSSION

From the theoretical standpoint, it would seem almost impossible to obtain from numbers and dimensions of glandular scales on what must always be practically a relatively extremely limited proportion of leaves anything more than an approximate indication of the richness of the herb in volatile oil. This is based on the following obvious facts and definite possibilities:

1. There is a continuous loss of volatile oil through the unbroken cuticle over the glandular scale, which must vary according to the atmospheric conditions. Hence, both quantity and quality of the residual secretions in the gland must vary considerably according to circumstances, without such differences being indicated in the external form or size of the gland.[9]
2. Doubtless there is a continuous production of volatile oil in the glandular scale, an entirely unpredictable factor.
3. The percentage of volatile oil in the glandular secretion very likely shows considerable fluctuations.
4. Rupture of the glandular scale wall and abscission of glands from mechanical and other causes constitute other indefinite variables.
5. Regeneration of cuticular wall after rupture and subsequent replenishment of secretion (which may occasionally occur) represent other unknown quantities.
6. Actual variation in distribution of glandular scales in the individual branch, plant, or part of the field may occur.[10]

For these reasons, it would appear that the factors involved are much too complex and variable to permit of a convenient, practical, and fairly accurate estimation of volatile oil yields on the basis of counts (and measurements) of glandular scales on what could never be more than an infinitesimal proportion of the entire plant crop.

This view is supported by the experimental findings. Unit area production of volatile oil showed rather poor correlation with the glandular scale counts made. Even though a fairly accurate correspondence might possibly be found between glandular scale counts on the one hand and oil yields on an herb weight basis on the other, yet this

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[7] Of the formula: formalin 37%, 135 cc.; acetic acid, glac., 65 cc.; alcohol, 50%, 2200 cc.

[8] Hesse (9) found slightly larger glandular scales on the upper leaves of peppermint in contrast to the lower.

[9] Brandt (10) demonstrated that the glandular head is not entirely filled by secretion, since on incision there is no tell-tale spurt to indicate internal pressures, even when this organ is apparently distended by secretion.

[10] If it is permissible to draw a parallel, attention at this point might be directed to the reported high degree of variation in potency of plants in the field, *e. g.*, belladonna (11).

would in itself really be of no practical significance without data on the herb yield per area.

As an example, a good stock of peppermint in the University of Florida Medicinal Plant Garden produced a fair yield of volatile oil (0.23% on a green basis) during the 1941 season (single harvest). The glandular scale counts ran 14 to 16 per sq. mm. Indiana-grown peppermint herb during the same season ran 15 to 19 glandular scales per sq. mm. (lower surface counts in both cases). Nevertheless, the Florida-grown mint showed a yield equivalent to only 5.99 lb. of oil per acre, whereas the yields in Indiana were generally in the neighborhood of 50 lb. per acre. The difference in yields in this case rests apparently not on deficiencies of the individual leaf so much as on poor vigor of the plants, as indicated in reduced aerial growth and lessened ramification of the shoots, together with poor coverage of the soil surface.

Rather, however, than attempt to correlate glandular scale counts with herbage yields, it would seem better from the practical viewpoint to carefully distill all the plants growing on several small measured representative areas, or even on a single area. This would be both a more accurate indicator and a really much less onerous procedure than by the proposed method of glandular scale estimation.

Microscopic examination of the leaf, either fresh or pressed dried, is nevertheless of use in roughly appraising mint material, certainly when compared with material of known quality.

SUMMARY

Experimental study has been made of the proposition that by examination of the glandular scales on a relatively few leaves in the mint field, one might possibly estimate the expected yield of volatile oil.

Methods of study are described both as to making counts of glandular scales and as to measurement of the same. Transparent adhesive cellulose tape for isolating the glandular scales from the leaf surface was found useful.

For comparative studies, counts were made of glandular scales on the lower surfaces of leaves which approached an arbitrary standard length. Leaves of peppermint growing in the Medicinal Plant Garden at Gainesville, Fla., summer of 1941, showed an average count per sq. mm. only slightly less than that for commercial Indiana peppermint of an excellent quality and high yield. The great differences in oil yield of the Florida (6 lb. per acre) and Indiana fields (around 50 lb. per acre) cannot therefore be attributed to discrepancies in the yield per leaf. It appears to depend at least partially on differences in size of the individual plants and in the utilization by the plant of the given growth area. The massive growth of the Indiana plant and its maintenance until harvest time must be considered the chief factor permitting a per acre yield about 1000% higher than for the plants growing in Florida.

A few tests were made to determine what effect if any on counts might be attributed to various physical factors of environment and handling. It was found that friction of the sort ordinarily encountered in manipulation of the herb is of practically no effect in effecting removal of glandular scales. Likewise, pressures up to at least 0.63 Gm./sq. mm. could be sustained without bursting or detaching glandular scales.

The count of glandular scales on the peppermint leaf varies considerably in accordance with several different factors, such as size, maturity, state of desiccation, surface (upper or lower), portion of lamina, environmental conditions of plant growth (nutrition, access to sunlight, latitude), etc. Counts on plants growing in the greenhouse were much lower than for plants outside, which corresponds with the much lower yield of oil in these plants.

It may safely be concluded from the experimental findings that glandular scale counts cannot be considered of practical utility, as compared to determination by direct distillation. It may reasonably be supposed that the approximate oil yield of the single leaf is of minor importance beside the variation in oil yield of the entire plant, as this is influenced by plant height, shoot ramification, etc.

Lateral dimensions of glandular scales were shown to vary considerably with location, being relatively greater when close to a large vein, on the upper (ventral) leaf surface, and especially when on the perianth. There were positive indications that glandular scales exposed to relatively more sunlight are of greater diameter than those receiving less. No correlation could be shown between average glandular scale diameter and oil content of leaf.

REFERENCES

(1) Hoelscher, Helena, and Bacon, F. J., *Am. Perfumer*, 25 (1930), 249; also THIS JOURNAL, 19 (1930), 624. Experimental data, however, did not appear here or in the M.A. thesis (unpublished) of Miss Hoelscher ("Histological Studies of Genus *Mentha*," Western Reserve Univ., Cleveland, O., May, 1930).

(2) Tunmann, O., *Ber. deut. pharm. Ges.*, 18 (1908), 491–540.

(3) Anon., *Pharm. Monatsh.*, 13 (1932), 27, 28.

(4) Tschirch, A., and Oesterle, *Anat. Atlas*, 1895–1900.

(5) Tschirch, A., and Tunmann, O., *Arch. Pharm.*, 239 (1901), 7–16.

(6) Tunmann, O., *Pharm. Ztg.*, 52 (1907), 353, 354.

(7) Pater, B., "Die Heilpflanzenversuchsanstalt der Landwirtschaftlichen Akademie in Kolozsvar," 1914, Heft 1, 47 pp.; through *Ann. Rept. Essential Oils, Synthetic Perfumes, etc., Schimmel & Co.*, 1914–1915, p. 65.

(8) Tschirch, A. Handbuch der Pharmakognosie. 1te. Aufl. Bd. 2. Teil 2. Leipzig: Verlag von Chr. Herm. Tauchnitz. 1917.

(9) Hesse, F., "Ueber die Groessenverhaeltnisse und Inhaltsbestandteile der Haare einiger offizineller Pflanzen." Diss. Wuerzburg: Druck Franz Standenraus, 1916, 61 pp.

(10) Brandt, W., *Proc. Int. Bot. Cong. (Fourth)*, Ithaca, N. Y., 1926, vol. II., 1362-*et seg.*

(11) Sievers, A. F., *J. Agr. Research*, 1 (1913), 129–146.

Elimination Studies on a Preparation of Injectio Digitalis U. S. P. XII*

By Marvin L. Pabst and George F. Cartland

The Twelfth Revision of the United States Pharmacopœia, which became official on November 1, 1942, lists for the first time a sterile aqueous solution of digitalis for intravenous use, "Injectio Digitalis." This had been under consideration for many years. In 1929, Hatcher and Haag (1) described a method of preparation which was recommended as a basis for further study should it be decided to admit an injectable digitalis to the U. S. P. The purpose of this paper is to report the results of studies on the rate of elimination following intravenous administration to cats of a sterile solution of digitalis prepared using the method outlined by Hatcher and Haag.

EXPERIMENTAL

Description of Digitalis Solutions.—The Injectio Digitalis[1] used in these studies meets the U. S. P. specifications and was prepared as follows: The dried and powdered leaves of *Digitalis purpurea* were extracted with distilled water. The aqueous extract was filtered and extracted with chloroform. The chloroform extract was concentrated to a small volume and precipitated by the addition of petroleum ether. The amorphous precipitate containing the cardioactive glucosides was dissolved in alcohol, diluted with water, clarified and assayed by the intravenous cat method (2). Phosphate buffer as recommended by Levy and Cullen (3) was added limiting the Na_2HPO_4 part so that the *p*H was between 6.5 and 7.0. The solution was diluted so that each cubic centimeter contained 0.33 U. S. P. Digitalis Unit, the alcohol adjusted to 10% and sterilized by Berkefeld filtration. The glucosidal complex assays approximately 1 U. S. P. Digitalis Unit per 1.8 mg. Although it is possible to prepare solutions more highly concentrated than 0.33 U. S. P. Digitalis Unit per cubic centimeter, such solutions are likely to develop troublesome precipitates in the ampul. The 10% alcohol serves as an excellent preservative and also helps to stabilize the solution against delayed precipitation.

Digitalis purpurea, as compared to *Digitalis lanata*, has offered greater difficulties in attempts to study its chemical composition by isolation and crystallization of the individual glucosides (5). However, the work of Cloetta (6) on a glucosidal fraction comparable to the one described here indicates that the chief active constituents are gitalin, bigitalin (gitoxin) and digitoxin.

* Received April 12, 1943, from the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

[1] Sterile Solution Digitalis—Upjohn lot 7984.

Since, in the preparation described above, yields are sacrificed to purity, it was considered desirable to make control elimination studies on the official Tincture of Digitalis U. S. P. XII which contains all of the active glucosides of dried digitalis leaves. This was prepared from official U. S. P. Digitalis Reference Standard, 1942. As an additional control, similar elimination studies were carried out on Solution Digilanid, N. N. R. (4) which is a glucosidal complex of *Digitalis lanata* in an aqueous solution containing alcohol 28% and glycerine 10%.

TABLE I. PRELIMINARY ELIMINATION STUDIES ON THREE DIGITALIS PREPARATIONS

Interval in Days	1	2	4	6	14
Sterile Solution Digitalis	40 (3)[a]	77 (5)	76 (3)	57 (2)	95 (3)
Tincture Digitalis U. S. P.	63 (2)	59 (2)	78 (2)	34 (2)	50 (2)
Digilanid N. N. R.	46 (3)	51 (2)	55 (2)	50 (2)	106 (2)[b]

[a] Per cent of initial dose eliminated during interval (number of cats in parentheses): Initial dose = 75% of average lethal dose.

[b] A cat above average in resistance to the digitalis preparation is responsible for this apparent elimination of greater than 100% of the initial dose.

Procedure.—The average lethal dose per Kg. was first determined for each of the digitalis preparations using the U. S. P. XII cat method (2). The cats were starved for 24 hr. and weighed. Under light ether anesthesia and using aseptic precautions 70% to 75% of the calculated fatal dose was injected into the femoral vein over a period of 2 to 3 min. The cats were kept in individual cages during the period allowed for digitalis elimination, starved during the final 24 hr. and again weighed. The average of the initial and final weights was used in calculating the final injection and the percentage elimination. At the end of the desired intervals the cats were killed by the intravenous injection of the digitalis solution allowing a period for the injection as near as possible to that specified by the U. S. P.

Each of the major series (Tables II, III, IV) was completed within a period of 17 days during which the average lethal dose per Kg. was again determined on a parallel group of 6 cats. The amount of digitalis solution eliminated was calculated for each cat by subtracting the calculated fatal dose based on parallel assay and average weight from the total volume of solution actually injected at the beginning and end of the interval.

Results.—Preliminary studies were carried out to estimate the percentage elimination of each of the preparations after intervals of 1, 2, 4, 6 and 14 days. The results are given in Table I with the number of cats indicated in parentheses. From the results obtained, it was decided to study the major series at intervals of 2, 6 and 14 days, the results of which are recorded in Tables II, III and IV. The mortality at the initial injection of 70% to 75% of the fatal dose was surprisingly low: 1 in 19 for Solution Digitalis lot 7984; 2 in 20 for Tincture Digitalis U. S. P.; 1 in 19 for Solution Digilanid N. N. R.

TABLE II.—ELIMINATION OF STERILE SOLUTION DIGITALIS LOT 7984

Cat No. and Sex	Initial Injection: Weight, Kg.	Initial Injection: Dose, Cc.	Final Injection: Interval, Days	Final Injection: Weight, Kg.	Final Injection: Average Weight, Kg.	Final Injection: Lethal Dose, Cc.	Fatal dose Calculated on Basis of Average Weight, Cc.	Total Injected, Initial and Final in Cc.	Amount Eliminated Over Interval in Cc.	Per Cent of Initial Dose Eliminated	Elimination during Period
93 F	1.76	2.36	2	1.64	1.70	1.43	3.11[a]	3.79	0.68	29	
94 F	1.84	2.46	2	1.76	1.80	1.85	3.29	4.31	1.02	41	
95 M	1.90	2.54	2	1.75	1.83	2.52	3.35	5.06	1.71	67	Average 63% in 2 days
105 F	2.44	3.26	2	2.23	2.34	4.08	4.28	7.34	3.06	94	
97 M	2.26	3.03	2	2.12	2.19	3.14	4.01	6.17	2.16	71	
98 M	1.76	2.36	2	1.64	1.70	2.54	3.11	4.90	1.79	76	
87 M	2.08	2.78	6	2.03	2.06	2.02	3.77	4.80	1.03	37	
88 F	2.12	2.84	6	2.03	2.08	1.68	3.81	4.52	0.71	25	
89 F	2.17	2.91	6	2.06	2.12	2.86	3.88	5.77	1.89	65	Average 40% in 6 days
90 F	2.18	2.92	6	2.04	2.11	1.51	3.86	4.43	0.57	20	
91 F	1.86	2.49	6	1.86	1.86	2.59	3.40	5.08	1.68	67	
92 F	2.12	2.84	6	2.08	2.10	1.68	3.84	4.52	0.68	24	
81 F	1.86	2.49	14	1.92	1.89	2.37	3.46	4.86	1.40	56	
82 M	1.86	2.49	14	1.96	1.91	3.66	3.50	6.15	2.65	106	
83 F	1.96	2.62	14	1.97	1.97	2.89	3.61	5.51	1.90	73	Average 72% in 14 days
84 F	2.02	2.70	14	2.00	2.01	2.04	3.68	4.74	1.06	39	
85 M	1.95	2.61	14	2.08	2.02	2.95	3.70	5.56	1.86	71	
86 F	1.80	2.41	14	1.88	1.84	3.06	3.37	5.47	2.10	87	

[a] In parallel assay the L. D. (average of 6 cats) was found to be 1.83 cc./Kg.

DISCUSSION

A comparison of Tables II and IV indicates a similar rate of elimination for the purified glucosidal fraction obtained from *Digitalis purpurea* (Solution Digitalis lot 7984) and that of *Digitalis lanata*

TABLE III.—ELIMINATION OF TINCTURE OF DIGITALIS U. S. P. XII
(Tincture prepared from U. S. P. Digitalis Reference Standard, 1942)

Cat No. and Sex	Initial Injection: Weight, Kg.	Initial Injection: Dose, Cc.	Final Injection: Interval, Days	Final Injection: Weight, Kg.	Final Injection: Average Weight, Kg.	Final Injection: Lethal Dose, Cc.	Fatal dose Calculated on Basis of Average Weight, Cc.	Total Injected, Initial and Final in Cc.	Amount Eliminated Over Interval in Cc.	Per Cent of Initial Dose Eliminated	Elimination during Period
143 F	2.44	1.11	2	2.16	2.30	0.92	1.40[a]	2.03	0.63	57	
144 F	2.34	1.06	2	2.14	2.24	0.95	1.37	2.01	0.64	60	
145 F	2.26	1.03	2	2.06	2.16	1.08	1.32	2.11	0.79	77	Average 55% in 2 days
156 M	2.02	0.92	2	1.84	1.93	0.70	1.18	1.62	0.44	48	
147 F	2.50	1.14	2	2.28	2.39	0.72	1.46	1.86	0.40	35	
148 F	1.82	0.83	2	1.68	1.75	0.66	1.07	1.49	0.42	51	
137 M	1.98	0.90	6	1.98	1.98	1.11	1.21	2.01	0.80	89	
138 F	2.16	0.98	6	2.22	2.19	0.64	1.34	1.62	0.28	29	
139 F	1.86	0.85	6	1.96	1.91	0.68	1.17	1.53	0.36	42	Average 54% in 6 days
140 F	2.42	1.10	6	2.40	2.41	0.83	1.47	1.93	0.46	42	
141 F	3.00	1.36	6	2.72	2.86	0.99	1.74	2.35	0.61	45	
142 M	2.90	1.32	6	2.82	2.86	1.42	1.74	2.74	1.00	76	
131M	2.08	0.95	14	2.10	2.09	1.11	1.27	2.06	0.79	83	
132 M	2.30	1.04	14	2.28	2.29	0.83	1.40	1.87	0.47	45	
133 F	2.36	1.07	14	2.34	2.35	0.80	1.43	1.87	0.44	41	Average 56% in 14 days
155 M	1.88	0.85	14	2.12	2.00	0.83	1.22	1.68	0.46	54	
135 F	1.96	0.89	14	1.98	1.97	0.67	1.20	1.56	0.36	40	
136M	2.16	0.98	14	2.12	2.14	1.04	1.31	2.02	0.71	72	

[a] In parallel assay the L. D. (average of 6 cats) was found to be 0.61 cc./Kg.

(Solution Digilanid N. N. R.). Both are eliminated to the extent of approximately 75% over a period of 14 days. However, the tincture prepared from the U. S. P. Digitalis Reference Standard, 1942 (Table III) is more persistent in its action since only slightly more than half of it is eliminated over a period of 14 days.

From Table II, the amount of Solution Digitalis lot 7984 eliminated is 63% in 2 days, but only 40% in 6 days. Irregularities such as this have also been observed by others (1, 7), and are considered to signify a cumulative digitalis effect but not necessarily an increase in the direct cardiac action.

SUMMARY

A preparation of Injectio Digitalis U. S. P. XII is described and its rate of elimination after intravenous injection in cats has been studied. Parallel control studies are reported on a Tincture of Digitalis prepared from the official U. S. P. XII Digitalis Ref-

TABLE IV.—ELIMINATION OF SOLUTION DIGILANID N. N. R.

Cat No. and Sex	Initial Injection: Weight, Kg.	Initial Injection: Dose, Cc.	Final Injection: Interval, Days	Final Injection: Weight, Kg.	Final Injection: Average Weight, Kg.	Final Injection: Lethal Dose, Cc.	Fatal dose Calculated on Basis of Average Weight, Cc.	Total Injected, Initial and Final in Cc.	Amount Eliminated Over Interval in Cc.	Per Cent of Initial Dose Eliminated	Elimination during Period
118 F	1.92	1.65	2	1.72	1.82	0.99	1.82[a]	2.64	0.82	50	
119 F	1.96	1.69	2	1.78	1.87	1.14	1.87	2.83	0.96	57	
121 F	2.06	1.77	2	1.80	1.93	1.62	1.93	3.39	1.46	82	Average 59% in 2 days
122 F	2.14	1.84	2	1.96	2.05	1.00	2.05	2.84	0.79	43	
123 M	2.50	2.15	2	2.32	2.41	1.32	2.41	3.47	1.06	49	
130 F	1.98	1.70	2	1.82	1.90	1.43	1.90	3.13	1.23	72	
112 F	2.02	1.74	6	1.86	1.94	1.05	1.94	2.79	0.85	49	
113 F	2.14	1.84	6	2.08	2.11	1.16	2.11	3.00	0.89	48	
114 F	2.14	1.84	6	2.14	2.14	1.85	2.14	3.69	1.55	84	Average 60% in 6 days
115 M	2.00	1.72	6	1.92	1.96	1.63	1.96	3.35	1.39	81	
116 F	1.94	1.67	6	1.80	1.87	1.00	1.87	2.67	0.80	48	
117 F	2.24	1.93	6	2.26	2.25	1.27	2.25	3.20	0.95	49	
106 F	1.82	1.57	14	1.80	1.81	1.75	1.81	3.32	1.51	96	
107 M	2.06	1.77	14	2.10	2.08	1.40	2.08	3.17	1.09	62	
108 F	2.10	1.81	14	1.90	2.00	1.53	2.00	3.34	1.34	74	Average 79% in 14 days
109 F	2.16	1.86	14	2.20	2.18	1.76	2.18	3.62	1.44	77	
110 M	1.80	1.55	14	1.94	1.87	1.52	1.87	3.07	1.20	77	
111 F	1.94	1.67	14	1.96	1.95	1.77	1.95	3.44	1.49	89	

[a] In parallel assay the L. D. (average of 6 cats) was found to be 1.00 cc./Kg.

erence Standard, 1942, and also on Solution Digilanid N. N. R. After the administration of approximately 75% of the lethal dose, the average elimination in 14 days, expressed in per cent of the initial dose, was 72% for Injectio Digitalis U. S. P. XII; 79% for Solution Digilanid N. N. R.; and 56% for the Tincture of Digitalis U. S. P. XII. Thus, the purified glucosidal preparations from *Digitalis purpurea* and *lanata* have similar rates of elimination but differ from the crude tincture which exerts a more prolonged effect.

REFERENCES

(1) Hatcher, R. A., and Haag, H. B., THIS JOURNAL, 18 (1929), 670.
(2) "Pharmacopœia of the United States," Twelfth revision, p. 510.
(3) Levy, R. L., and Cullen, G. E., *J. Exptl. Med.*, 31 (1920), 267.
(4) "New and Non-official Remedies," 1942, p. 270.
(5) Stoll, A., THIS JOURNAL, 27 (1938), 761.
(6) Cloetta, M., *Arch. exptl. Path. Pharmakol.*, 112 (1926), 261.
(7) Gold, H., *Arch. Internal Med.*, 32 (1923), 779.

A Preliminary Study of *Piper marginatum* Jacq.*

By Emilia Hoyo de Núñez and Carl H. Johnson†

Piper marginatum Jacq. (Fam. *Piperaceae*) is a Puerto Rican plant to which a number of medicinal properties have been attributed. de Grosourdy (1) stated that the leaves of this plant act as a hemostatic in cases of traumatic hemorrhages, lesions, epistaxis, hemoptisis and in uterine hemorrhages. He also claimed that in diarrheas and in the chronic dysenteries so common in the tropics an infusion of the leaves is very effective.

The present investigation was carried out to determine the active constituents present in the leaves, especially those responsible for the medicinal value of the plant. A review of the literature revealed that no phytochemical work has been reported on this species, all the work recorded being of a botanical nature.

The leaves used in this investigation were collected in Puerto Rico during October and December of 1940. They were air-dried on screens in a well-ventilated room and then were ground to a No. 60 powder. The ground material was of a dark green color with a very aromatic odor and, on rubbing between the fingers, left a greasy film suggesting the presence of a fatty substance.

EXPERIMENTAL

GENERAL INVESTIGATION

In a general study using the methods of the United States Pharmacopœia, Eleventh Revision, the following results were obtained:

Total ash	10.69%
Acid-insoluble ash	2.91%
Moisture, oven method	14.66%
Moisture, toluene method	8.55%

By treatment of a 20-Gm. sample in a Soxhlet apparatus with the solvents listed in succession the following amounts of extractives were obtained:

Petroleum benzin	14.99%
Ether	1.55%
Chloroform	2.16%
Absolute alcohol	10.47%
Dilute alcohol	13.46%
Water	6.00%
Total extractives	48.63%

An aqueous extract of a small amount of the material gave a positive reaction for tannin with ferric chloride T. S. and for reducing sugars with Fehling's solution. An acidulated water extract reacted positively with the alkaloidal reagents, iodine T. S. and mercuric potassium iodide T. S. Tests for starch and saponins in the solid material gave negative results.

DETAILED INVESTIGATION

The Stas-Otto Process.—Fifty grams of the material was heated with alcohol acidified with tartaric acid, filtered, evaporated, dissolved and again evaporated. When this residue was dissolved in acid water and shaken with ether no alkaloids

* This paper is based on a thesis presented to the Graduate Council of the University of Florida, Gainesville, Fla., in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

Received Sept. 4, 1942.

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be obtained. However, when the aqueous solution was made alkaline with sodium hydroxide then ether could extract substances giving a positive test with several alkaloidal reagents, as was also possible with chloroform after the alkaline liquid was made ammoniacal. But, the aqueous solution left after shaking out thoroughly with each solvent still contained some of these reacting substances. Therefore ether and chloroform are not appropriate solvents.

Tannin.—By the general method of the Assoc. Official Agr. Chem. (2), 0.69% tannin, expressed as gallotannic acid, was obtained, while by the hide powder method (3) only 0.28% was found.

Volatile Oil.—A 50-Gm. portion was subjected to the volatile oil determination method of the Assoc. Official Agr. Chem. (4). On the basis of the air-dried leaves the material contained 6.97% of volatile oil with the following properties:

Color	colorless, turning yellow
Odor	sassafras-like
Taste	mint-like
Specific gravity, 25° C.	1.0560
Refractive index, 20° C.	1.5475
Specific rotation, $\frac{30° \text{ C.}}{D}$	+0.52°

On freezing to −78° C. the oil slowly formed a thick sticky mass without showing the usual phenomena of congealing. A small amount of the air-dried fruits yielded 7.62% of volatile oil resembling that from the leaves.

The Lead Method.—The procedure of Rosenthaler (5) was carried out. Two hundred grams of the ground leaves was boiled with water, cooled, filtered, and the liquid treated with lead acetate solution to get precipitate *A*, which was brown and curdy. Upon removal of this, the filtrate was treated with basic lead acetate to yield precipitate *B* having a yellow color. After separating and washing the latter, the liquid part was labeled filtrate *B*.

In the alcohol extract of precipitate *A* a color with ferric chloride T. S. indicated a tannin and a positive reaction with Fehling's solution showed a reducing substance. Upon precipitating the lead as the sulfide and treating the latter with alcohol some fine crystalline needles melting at 105° C. were obtained. These were too small in amount to permit further work.

Upon liberation and removal of the lead from precipitate *B* carbohydrates were found according to the alpha naphthol test. By using Benedict's solution and other reagents it was found that reducing sugars were present in the carbohydrates.

The water extract of the lead sulfide formed by the treatment of precipitate *B* with hydrogen sulfide gave a positive test with alkaloidal reagents. Alkaloids could not be extracted by immiscible solvents. Further treatment of the lead sulfide precipitate with ammonia yielded a crystalline mixture consisting of 2 substances melting at 194° and 214° C. The amount was too small for further investigation.

A portion of filtrate *B*, after being freed from lead, was treated with immiscible solvents as in the Stas-Otto process but no alkaloids could be found. This filtrate yielded crystals melting at 250° C. that did not reduce Fehling's solution.

Aqueous and alcoholic extracts of the lead sulfide precipitate obtained from filtrate *B* gave positive tests with alkaloidal reagents but the substance could not be separated. An ammonia extract left crystals melting at 258° C., but the amount was very small.

Large-Scale Extraction.—In order to make a more complete examination of the leaves 500 Gm. of the powdered material was treated with various solvents after the volatile oil had been removed. The dry material was packed in a modified Wester extraction apparatus (6) and percolated with petroleum benzin, ether, chloroform and absolute alcohol in succession.

The oily, semisolid, petroleum benzin extract was divided into 2 portions by treatment with ethyl acetate according to the method of Cwalina and Jenkins (7). The unsaponifiable portion of the ethyl acetate-soluble material responded to the usual tests for phytosterol given by Rosenthaler (8) but none could be crystallized out. The saponifiable portion yielded a crystalline substance melting at 100° C. which was probably a fatty acid.

From the ethyl acetate-insoluble material of the petroleum benzin extract the unsaponifiable portion yielded small quantities of white scales melting at 120° C. and a cream-colored powder melting at 57° C. The saponifiable portion yielded a white powder melting at 70° C. which was probably a fatty acid.

The ether extract was a greasy, dark greenish material. This residue contained substances that reacted with alkaloidal reagents but which could not be extracted with immiscible solvents. Tannins were present.

The chloroform extract was a thick, greasy, dark brown mass. On prolonged shaking with ether, alkaloid-like material could be obtained as indicated by Mayer's reagent.

With absolute alcohol a thick brown extract containing the substance giving a positive test with alkaloidal reagents was obtained. Of various organic solvents, only butyl and amyl alcohols could extract any appreciable amount of this reacting substance.

DISCUSSION

In this plant is present a substance that reacts with the usual alkaloidal reagents but cannot easily be shaken out like most alkaloids. Further work is necessary to determine whether this substance is an alkaloid. It is soluble to some extent in alcohol and water, slightly soluble in amyl and butyl alcohols, but difficultly soluble in ether, chloroform, petroleum benzin, ethyl acetate, benzene and xylene.

The discrepancy between the moisture content by the oven and toluene methods is probably due, to a great extent, to the volatile oil which is lost during the heating in the oven.

A few preliminary pharmacological experiments indicated that the fraction of the drug reacting with alkaloidal reagents may possess a depressant action on smooth muscle. However, further work is necessary to prove this point. An apparent reduction in coagulation and bleeding times also requires more complete investigation.

SUMMARY

The dried leaves of *Piper marginatum* contain 6.97% of volatile oil, 0.28% of tannin, also reducing sugars, a phytosterol, a fat, an unknown substance resembling an alkaloid believed to be the active constituent, and very small quantities of some unidentified crystalline substances.

REFERENCES

(1) de Grosourdy, D. R., "El Medico Botanico Criollo," Libreria de Francisco Brachet, Paris, 1864, Vol. III, p. 71.

(2) "Methods of Analysis," Assoc. Official Agr. Chem., Washington, D. C., Fourth edition, 1935, p. 196.

(3) *Ibid.*, p. 117.

(4) *Ibid.*, p. 447.

(5) Rosenthaler, L., "The Chemical Investigation of Plants," Translated by Ghosh, S., G. Bell and Sons, Ltd., London, Third edition, 1930, p. 31.

(6) *Ibid.*, p. 190.

(7) Cwalina, G. E., and Jenkins, G. L., THIS JOURNAL, 27 (1938), 585.

(8) Rosenthaler, L., *loc. cit.*, p. 78.

A Study of the Assays of the Powdered Extracts of Certain Solanaceous Drugs*

By H. Virginia Gilliland†, K. L. Kaufman‡ and H. Bang§

There is an extensive literature dealing with the problem of a satisfactory assay for the most important members of the *Solanaceae*, namely, belladonna and hyoscyamus. Because of their importance it has been necessary for the pharmaceutical chemist to make every effort to determine exactly the potency of these drugs. The work herein presented has been an attempt to locate the origin of some of the difficulties in the assays of the powdered extracts and to show a comparison of the original U. S. P. XI and the U. S. P. XI Second Supplement methods of assay.

It is the general consensus that the conflicting reports on the alkaloidal content of these drugs may be traced to volatile bases which have not been included in the assay results in some cases and in others have been calculated as alkaloid (1, 2, 3, 4, 5). As early as 1870, Hohn (6) reported the presence of a base resembling methylamine in hyoscyamus seed. Lefort (7), Puckner (8), Goris and Larsonneau (9), Aoyama (10), Markwell and Walker (2) and Ewe (5) also mentioned the presence of volatile ammoniacal substances. Schaller and Baldinger (11) and Hester and Davy (12) stated that these ammoniacal substances were ammonia soaps. Evans and Davy (4) stated both ammonia and amines were present. In 1934, DeKay and Jordan (3) confirmed the presence of choline in hyoscyamus and stated that the variable results might be due to more choline being decomposed in some assays than in others. They identified trimethylamine and possibly dimethylamine in the chloroform extract of this drug. In 1937, the Subcommittee on Mydriatic Alkaloidal Assays of the A. D. M. A. Contact Committee (13) found that the physiological activity of these volatile bases ranged from none to one-twentieth that of atropine sulfate. Since

* Based on a thesis submitted to the Committee on Graduate Studies of the State College of Washington, Pullman, Wash., by H. V. Gilliland in partial fulfillment of the requirements of the degree of Master of Science, June, 1941.

Presented to the Scientific Section of the A. PH. A., Denver meeting, 1942.

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the mydriatic activity of these bases was so weak and variable, the Committee recommended they be removed by the heat treatment.

There are few reports regarding the decomposition of the alkaloidal content with age. Ortlieb (14) found that the alkaloidal value of belladonna and hyoscyamus extracts diminished on keeping. Ribaut (15) on reëxamination of a number of extracts after 4 yr. found that their value had diminished materially. Fricotel (16) and Jacobsen (17) have also reported retrogression of alkaloidal content and great changes in solubility on standing. Other investigators have reported contrary results. Gaze (18), Thaysen (19) and Carlinfanti (20) all reported that there was no variation or decrease in alkaloidal content on standing over a period of about $2^1/_2$ yr. Despite these, Jendrassik and Will (21) report that older extracts give only a fraction of the effect of fresh extracts, when assayed by a physiological method which gives results comparable to the chemical method.

The assay methods for the powdered extracts of belladonna and hyoscyamus were very similar until the presence of volatile bases was recognized and a method proposed to eliminate them, which led to the U. S. P. XI method. This was replaced by a revised procedure official in the U. S. P. XI Second Supplement. The essential difference between the 2 methods lies in the initial extraction step. The U. S. P. XI specified digestion of the extract in dilute sulfuric acid and chloroform; filtration through purified cotton and washing the residue with weak acid. The Supplement Method requires maceration of the extract overnight in a mixture of alcohol, ether and ammonia water, followed by extraction of the sample for 3 hr. with ether. The ether extract is then extracted with sulfuric acid, and the 2 methods thereafter are essentially identical.

TABLE I.—POWDERED EXTRACT OF BELLADONNA

Samples	U. S. P. XI Supplement		U. S. P. XI	
	Gm. Alkaloids per 100 Gm. Experimentally	Per Cent Return of Alkaloids	Gm. Alkaloids per 100 Gm. Experimentally	Per Cent of U. S. P. Average
A (Manufactured in 1937, opened)	1.079	86.32	0.9811	78.48
	1.112	88.96	0.9918	79.35
	1.115	89.20	0.9997	79.98
	1.121	89.67	1.0185	81.48
	1.160	92.80	1.0233	81.85
	Av. = 1.1174	Av. = 89.39	1.0390	83.15
			Av. = 1.0089	Av. = 80.72
B (Manufactured in 1937, unopened)	1.080	86.40	0.9129	73.03
	1.109	88.72	0.9189	73.51
	1.620	90.85	0.9395	74.36
	Av. = 1.1166	Av. = 88.66	0.9545	76.36
			Av. = 0.9307	Av. = 74.45
C (Manufactured in 1940, unopened)	1.304	104.32	1.080	86.40
	1.310	104.80	1.081	86.48
	1.315	105.20	1.081	86.48
	1.317	105.36	1.087	86.96
	1.327	106.18	Av. = 1.0822	Av. = 86.55
	1.329	106.32		
	Av. = 1.317	Av. = 105.32		

EXPERIMENTAL

Comparison of U. S. P. XI and U. S. P. XI Second Supplement Methods of Assay.—A comparison of the 2 methods was made upon 3 samples of powdered extract of belladonna and upon 2 samples of powdered extract of hyoscyamus. The percentages listed in the last column of Tables I and II are based upon the average alkaloidal content of U. S. P. XI extracts of belladonna and hyoscyamus or 1.25 Gm. of alkaloid per 100 Gm. of belladonna extract and 0.155 Gm. of alkaloids per 100 Gm. of hyoscyamus extract. The results of the comparison are shown in Tables I and II. An analytical grade of chloroform was used.

Effect of Age Upon Alkaloidal Content of Extracts.—The effect of age was suggested as a possible explanation for low results in some experimental work, consequently old and fresh samples of powdered ex-

tracts were assayed by the U. S. P. XI method with the use of analytical chloroform to determine this effect. It was assumed that the old extracts, manufactured in 1936 and 1937, were adjusted by this method.

TABLE II.—POWDERED EXTRACT OF HYOSCYAMUS

Samples	U. S. P. XI Supplement: Gm. Alkaloids per 100 Gm. Experimentally	U. S. P. XI Supplement: Per Cent Return of Alkaloids	U. S. P. XI: Gm. Alkaloids per 100 Gm. Experimentally	U. S. P. XI: Per Cent of U. S. P. Average
A (Manufactured in 1936, unopened)	0.1674	108.00	0.1567	100.11
	0.1699	109.61	0.1652	106.58
	0.1724	111.23	0.1709	110.28
	0.1732	111.74		
	Av. = 0.1707	Av. = 110.14	Av. = 0.1643	Av. = 105.66
B (Manufactured in 1940, unopened)	0.1709	110.02	0.1743	112.44
	0.1745	112.56	0.1745	112.56
	0.1790	115.47	0.1752	113.01
	0.1790	115.47	0.1756	113.90
	Av. = 0.1758	Av. = 113.38	Av. = 0.1751	Av. = 112.98

Correspondence with the manufacturer indicated they were U. S. P. strength at the time of manufacture. The fresh samples which served as controls were of 1940 manufacture and were of U. S. P. strength when assayed by the Supplement method. For this effect see Tables I and II under the heading "U. S. P. XI" method of assay.

A sample of a "synthetic extract" used by Fricke and Kaufman (22) containing only arrowroot starch, atropine and chlorophyll was available. It had been stored under the same conditions as the powdered extracts of belladonna and hyoscyamus (cool, dark and dry atmosphere). A fresh sample, identical in composition, was prepared, and the 2 were assayed by the Supplement method, using analytical chloroform. The results are shown in Table III. The old sample was manufactured in 1937 and assayed 99.28% atropine at that time.

Effect of Added Amines to Powdered Extracts.—An attempt was made to determine the effect, if any, of adding di- and trimethylamine hydrochlorides to synthetically prepared extracts. This work is a continuation of that begun by Fricke and Kaufman (22) who determined the effect of diluents and of chlorophyll. These amines were chosen because they have been reported as being present in the drugs under examination. Individual samples were prepared in the following manner:

Approximately 2 Gm. arrowroot starch was accurately weighed and placed in a glass mortar. To this was added 2 drops of chlorophyll and the mixture was triturated until a uniform color resulted. Weighed amounts of atropine (about 0.026 Gm.) and the amine hydrochloride (weighed by difference) were then added and the whole triturated very thoroughly. The sample was then transferred to a clean, dry test tube, stoppered and

TABLE III.—EFFECT OF AGE UPON SYNTHETIC EXTRACT

Sample	U. S. P. XI Supplement Method: Gm. Alkaloids Calcd. from Titration Values. Experimentally	U. S. P. XI Supplement Method: Gm. Alkaloids Actually Present	Per Cent
3 yr. old	0.02272		
	0.02283		
	0.02433		
	Av. = 0.02329	Av. = 0.02512	92.72
Fresh	0.02455		
	0.02491		
	0.02503		
	Av. = 0.02479	Av. = 0.02494	99.40

Effect of Grade of Chloroform on Assay.—The effect of grade of chloroform has previously been stated by Fricke and Kaufman (22) as having an effect upon the assay results. In this work technical, analytical and U. S. P. XI grades were used. *Technical chloroform was found to give results which were of no value.* Because of the difference in price of the Analytical and U. S. P. XI grades of chloroform, a comparison was made of these to determine their relative efficiency. Assays were made using both belladonna and hyoscyamus extracts. The results are shown in Table IV.

sealed with paraffin. The assays were run immediately following the preparation of one series of samples. The paraffin was removed very carefully from the test tube and each individual sample was weighed before analysis. From the weight of the sample as prepared and as weighed for analysis, the amount of atropine present in the sample was calculated.

The first series of samples containing trimethylamine hydrochloride were assayed by a short method as proposed by Fricke and Kaufman (22), assayed by the Supplement method. The results shown in Table VI indicate that the heat treatment did not remove this amine.

TABLE IV

Sample and Method	Analytical Chloroform: Gm. Alkaloids per 100 Gm. Experimentally	Analytical Chloroform: Per Cent Return of Alkaloids	U. S. P. XI Chloroform: Gm. Alkaloids per 100 Gm. Experimentally	U. S. P. XI Chloroform: Per Cent Return of Alkaloids
C 1940				
Powdered extract belladonna, Supplement Method	1.304	104.32[a]	1.310	104.80[a]
	1.327	106.16[a]	1.315	105.20[a]
	1.329	106.32[a]	1.317	105.36[a]
	Av. = 1.320	Av. = 105.60[a]	Av. = 1.314	Av. = 105.12[a]
C 1940				
Powdered Extract Belladonna, U. S. P. XI Method	1.0810	86.48[a]	1.0800	86.40[a]
	1.0810	86.48[a]	1.0870	86.86[a]
	Av. = 1.0810	Av. = 86.48[a]	Av. = 1.0835	Av. = 86.68[a]
B 1940				
Powdered Extract Hyoscyamus, Supplement Method	0.1709	110.02[b]	0.1698	109.55[b]
	0.1745	112.56[b]	0.1703	109.85[b]
	0.1790	115.47[b]	0.1717	110.74[b]
	0.1790	115.47[b]		
	Av. = 0.1758	Av. = 113.38[b]	Av. = 0.1706	Av. = 110.04[b]
B 1940				
Powdered Extract Hyoscyamus, U. S. P. XI Method	0.1743	112.44[b]	0.1661	107.17[b]
	0.1745	112.56[b]	0.1663	107.27[b]
	0.1752	113.01[b]	0.1718	110.83[b]
	0.1766	113.90[b]		
	Av. = 0.1751	Av. = 112.98[b]	Av. = 0.1681	Av. = 108.43[b]

[a] Per cent return of belladonna calculated using 1.25 Gm. alkaloids/100 Gm. extract as 100%.
[b] Per cent return hyoscyamus calculated using 0.155 Gm. alkaloid/100 Gm. extract as 100%.

U. S. P. XI, and U. S. P. XI Supplement, to check the suitability of the 3 methods. The results are shown in Table V:

TABLE V.—EFFECT OF TRIMETHYLAMINE HYDROCHLORIDE

Methods	Gm. of Trimethylamine	Gm. of Atropine	Gm. of Alkaloid Found (Calcd. as Atropine)
Short	0.0394	0.02738	0.01307
	0.0208	0.02800	0.01433
	0.0138[a]	0.02683[a]	0.03584[a]
U. S. P. XI	0.0186	0.02945	0.02456
	0.0184	0.02873	0.02422
	0.0188[a]	0.02877[a]	0.03011[a]
U. S. P. XI, Second Supplement	0.0228	0.02828	0.02728
	0.0196	0.02520	0.02357
	0.0144[a]	0.02878[a]	0.03104[a]

[a] No heat treatment used.

Data in Table V show the short method was unsuitable for the assay of synthetic extracts containing amines, and confirmed the results of the U. S. P. XI and Supplement methods on the Powdered Extracts. The heat treatment served to remove this amine.

A second series of individual samples containing dimethylamine hydrochloride were prepared and

The data in Tables V and VI raised the question of the effect of adding these amines to a powdered extract of belladonna. The extract chosen was a

TABLE VI.—EFFECT OF DIMETHYLAMINE, SUPPLEMENT METHOD

Gm. of Dimethylamine	Gm. of Atropine	Gm. of Alkaloid Found (Calcd. as Atropine)
0.0650	0.02837	0.05578
0.0430	0.03074	0.05670
0.0290	0.03139	0.05537
0.0360	0.02935	0.05468
0.0232[a]	0.03057[a]	0.061468[a]
0.0314[a]	0.04284[a]	0.05468[a]

[a] No heat treatment used.

fresh one whose potency had been previously determined. Three series of samples were prepared by adding atropine, dimethylamine hydrochloride and trimethylamine hydrochloride to weighed samples of the extract. The assays were made using the Supplement method with U. S. P. XI chloroform. The results are shown in Tables VII and VIII.

Tests on Chloroform.—It was thought that the reason for the excessive destruction of alkaloids noted using technical chloroform was due to the presence of phosgene. However, suitable tests

TABLE VII.—EFFECT OF ADDED ATROPINE

Sample	Extract Alone: Gm. Alkaloid Calcd. from Titration Values	Extract Alone: Per Cent Return of Alkaloids[a]	Atropine Added: Gm. of Atropine Added	Atropine Added: Gm. Atropine from Titration Values, over and above That from Sample	Atropine Added: Per Cent Return Added Atropine
C 1940	0.02641	104.80	0.0279	0.02748	98.50
	0.02636	104.20	0.0501	0.04893	98.67
	0.02641	105.36	0.0323	0.03230	99.57
	Av. = 0.02639	Av. = 105.12			Av. = 98.58

[a] Per cent return calculated using 0.025 Gm. alkaloid/2 Gm. extract as 100%.

(23, 24, 25, 26), showed phosgene present in all grades. Technical and analytical chloroform was shaken out with water which had recently been twice distilled from glass and the *p*H of the resulting aqueous extracts was determined potentiometrically. Results were as follows:

Extract from Technical Chloroform..*p*H 8.62
Extract from Analytical Chloroform..*p*H 6.59
Double Distilled Water.....*p*H 6.514

TABLE VIII.—EFFECT OF ADDED DIMETHYLAMINE AND TRIMETHYLAMINE HYDROCHLORIDE

Sample	Gm. Alkaloids Calcd. for Sample[a] Using 0.025 Gm./2 Gm. as Standard	Gm. Amine Added to Samples	Gm. Alkaloids Calcd. as Atropine from Titration Values	Per Cent of Alkaloids Returned
C 1940	0.01906	0.0384	0.01994	104.62
Trimethylamine	0.01896	0.0296	0.01902	100.32
	0.01879[a]	0.0320[a]	0.02780[a]	147.95[a]
C 1940	0.01910	0.0392	0.01954	102.30
Dimethylamine	0.01918	0.0602	0.01994	103.90
	0.01880[a]	0.0494[a]	0.02295[a]	122.03[a]

[a] No heat treatment used.

DISCUSSION AND SUMMARY

1. The U. S. P. XI Second Supplement method for the assay of the powdered extracts of belladonna and hyoscyamus is superior to the U. S. P. XI method for the following reasons:

(*a*) The Supplement method returned on the average 99% of the alkaloids, which is a 10% to 15% higher return of alkaloids than is obtained using the U. S. P. XI method.

(*b*) The Supplement method is easier to use than the U. S. P. XI method because extractions are performed more rapidly and are complete.

(*c*) The Supplement method takes no more time of the operator to complete than does the U. S. P. XI method.

(*d*) When applied to "synthetic extracts" containing amines, the Supplement method gave results approaching the theoretical.

2. Technical grade of chloroform is unsuitable for alkaloidal determinations. Hydrogen ion determination indicates that it contains alkaline elements, and the destruction noted is probably due to this. Comparisons of Analytical and U. S. P. XI grades of chloroform gave results that were in close agreement and show the suitability of the latter grade for the assay of these preparations.

3. From experimental results there was evidence of a deterioration of the extracts with age, but no definite statement can be made since the manufacturer's complete records were not available. A 3-yr.-old "synthetic" sample was compared to a fresh sample and was found to be 7% deficient in alkaloidal content. Since Fricke and Kaufman (22) showed there was little, if any, adsorption of the alkaloids by the starch, it was believed that the age of the extract did have some effect upon the assay results, confirming the reports of Ortlieb (14), Ribaut (15), Fricotel (16), Jendrassik and Will (21), and Vincke and Oelkers (27).

4. The addition of trimethylamine to synthetically prepared extracts and to the powdered extract of belladonna showed it was removed by the heat treatment as specified in the Supplement method of assay. The addition of dimethylamine to a

synthetically prepared extract showed that it was not removed by the heat treatment, but when added to the powdered extract of belladonna, the heat treatment served to remove it. No attempt was made to explain the behavior of dimethylamine.

REFERENCES

(1) Anonymous, *Proc. Am. Pharm. Assoc.*, 57 (1909), 75.

(2) Markwell, W. A. N., and Walker, J., *Chemist and Druggist*, 109 (1928), 525; *Yearbook Am. Pharm. Assoc.*, 18 (1929), 458.

(3) DeKay, H. G., and Jordan, C. B., THIS JOURNAL, 23 (1934), 316, 391.

(4) Evans, M. D., and Davy, E. D., *Ibid.*, 23 (1934), 388.

(5) Ewe, G. E., *Ibid.*, 21 (1932), 870.

(6) Hohn, H., *Pharm. J.*, (3), 30 (1870–1871), 304.

(7) Lefort, M. J., *Ibid.*, (3), 32 (1872–1873), 53.

(8) Puckner, W. J., *Am. J. Pharm.*, 80 (1908) 66.

(9) Goris, A., and Larsonneau, A., *Bull. sci. pharmacol.*, 28 (1921), 499; *C. A.*, 16 (1922), 787.

(10) Aoyama, S. J., *Pharm. Soc. Japan.*, 48 (1928), 150; *C. A.*, 22 (1928), 2360.

(11) Schaller, N. C., and Baldinger, L. H., THIS JOURNAL, 21 (1932), 442.

(12) Hester, E. E., and Davy, E. D., *Ibid.*, 22 (1933), 514.

(13) *Proc. Am. Drug. Manuf. Assoc.*, The Baltimore Press, Baltimore, Maryland, 1937, pp. 145–165.

(14) Ortlieb, G., *Pharm. Ztg.*, 48 (1903), 162; *Proc. Am. Pharm. Assoc.*, 51 (1903), 621.

(15) Ribaut, H., *Bull. sci. pharmacol.*, 9 (1908); *Ibid.*, 57 (1909), 74.

(16) Fricotel, J., *Ibid.*, 15 (1908), 687; *C. A.*, 4 (1910), 234.

(17) Jacobsen, K. A., *Apoth.-Ztg.* 26 (1911), 422; *Proc. Am. Pharm. Assoc.*, 59 (1911), 54.

(18) Gaze, R., *Apoth. Ztg.*, 27 (1911), 402; *C. A.*, 6 (1912), 2284.

(19) Thaysen, H., *Ibid.*, (1912), 528; *C. A.*, 7 (1913), 681.

(20) Carlinfanti, E., *Boll. chim.-farm.*, 51 (1912), 777; *C. A.*, 7 (1913), 1400.

(21) Jendrassik, L., and Will, G., *Arch. exptl. Path. Pharmakol.*, 153 (1930), 94.

(22) Fricke, H. H., and Kaufman, K. L., THIS JOURNAL, 28 (1939), 215.

(23) Dyson, G. M., *Chem. Revs.*, 4 (1937), 132.

(24) *Chem. Ztg.* 16 (1892), 1230; *Merck's Index* (1940), 873.

(25) *Z. anal. Chem.*, 108 (1937), 24; *Merck's Index* (1940), 889.

(26) *Apoth. Ztg.* 28 (1913), 709; *Merck's Index* (1940), 662.

(27) Vincke, E., and Oelkers, H. A., *Med. Klin.*, 33 (1937), 80; *Pharm. Abstracts*, 3 (1937), 263

Assay of Oregon Ergot*

By E. T. Stuhr, B. E. Christensen and Edgar Wong

In view of the existing world conditions the question of an adequate supply of ergot is very pertinent at this time. The bulk of this drug has in the past been imported from Continental Europe. This former dependence on European markets may be attributed to high labor costs, inferior quality of the local product, and poor stands of this fungus. Since the need for ergot is essential, potential domestic sources are being investigated.

Infestation of *Claviceps purpurea* (Fries) Tulasne on wild ryegrass in the Oregon country was previously reported in 1931 (1). It has been observed that the grasses of the range land of southeastern Oregon are frequently infested with ergot. The extent of infestation varies from year to year. During 1940 and 1941 the grasses throughout Harney county were extensively infested—particularly the giant wild ryegrass, the blue joint grass, and the Nevada bluegrass. (See Figs. 1 and 2.)

Considering the rather abundant stand of ergot in Oregon it offers some promise as a potential source of supply. As the shortage

* A coöperative study from the Department of Chemistry and the Department of Pharmacology and Pharmacognosy, Oregon State College, Corvallis, Ore.

Received Jan. 20, 1943.

Fig. 1.—Infested blue joint grass.

of this drug may become acute, samples of last year's (1941) growth from Harney county were assayed by both chemical and biological methods.

EXPERIMENTAL

The ergot was collected and hand-picked during the summer of 1941. The curing and storage of this crude ergot, and the processing and preparation of the fluidextract were in accordance with U. S. P. XI specifications (2).

Chemical Assay by Colorimetric Method.—Samples of both crude ergot and fluidextract were compared colorimetrically with commercial samples according to the method of Allport and Jones (3). The results of these assays are given in Table I.

Bioassay by Cock's Comb Method.—To determine the relative activity of the Oregon ergot, a bioassay (cock's comb method) of the fluidextract was under-

taken. The cockerels were standardized, using ergotoxine ethanesulfonate as the standard (2).

TABLE I.—COMPARISON OF OREGON ERGOT WITH COMMERCIAL SAMPLES

Sample	Standards* (U. S. P. Quality)	Relative concentrations of Oregon Ergot: Total Alkaloid	Ergotoxine	Ergonovine (Ergometrine)
Fluid	Commercial fluidextract	3.1	4.1	1.6
Solid A	Commercial crude ergot	3.5	3.8	3.1
Solid B	Commercial crude ergot	2.2	1.9	3.0

* Concentration of standards 1.0.

The results as summarized in Table II indicate that the fluidextract possessed a relative activity (potency) of from 250 to 300+.

DISCUSSION

The Oregon ergot produced characteristic physiological reactions and the fluidextract possessed a pronounced potency.

The bioassay findings correlate favorably with the colorimetric assay.

SUMMARY

1. Extensive ergot infestation in southeastern Oregon has been reported.

2. A comparison of the Oregon ergot to commercial samples was made colorimetrically.

3. The relative activity of the Oregon ergot was determined by the cock's comb method.

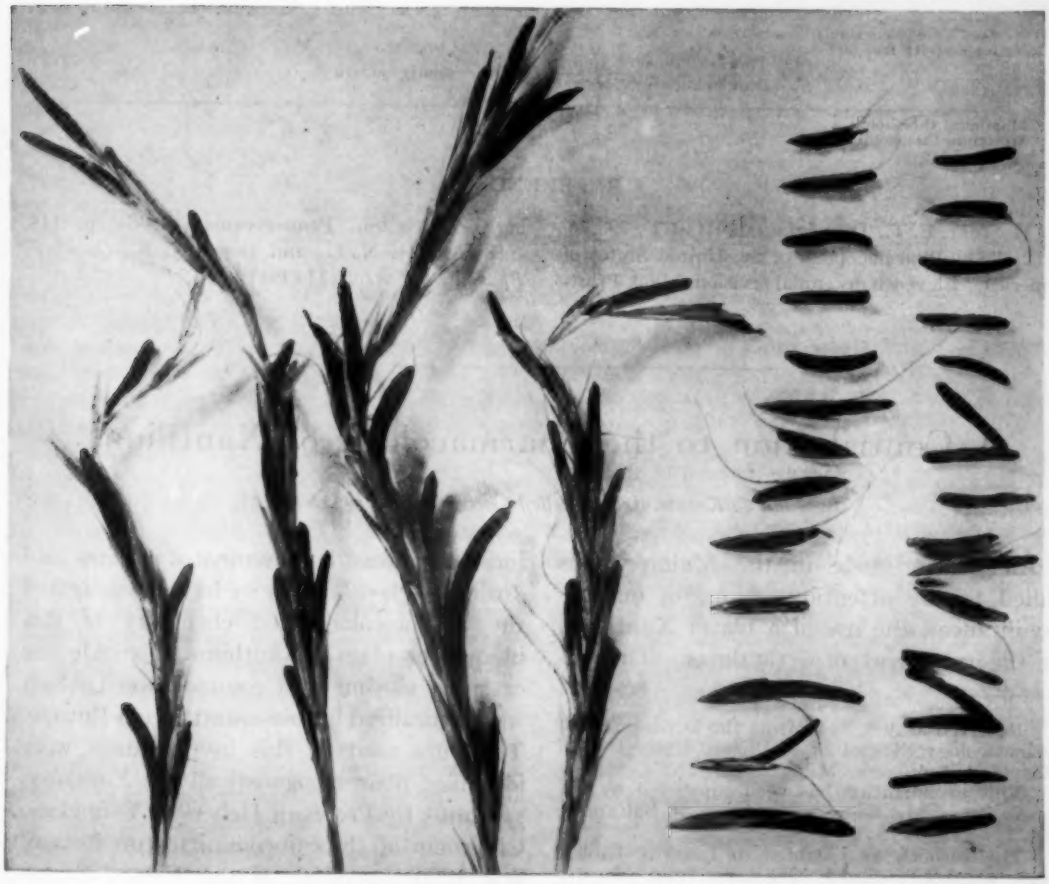

Fig. 2.—Infested giant wild ryegrass.

TABLE II.—ASSAY OF OREGON ERGOT RAPIDITY OF ONSET AND INTENSITY OF EFFECTS

Series A Cock	Weight in Gm.	Cc./Kg.	Reaction Time	Intensity of Effect
1	1301	0.30	6–8 min.	Pronounced coloration
			1 hr.	Excessive cyanosis
			24 hr.	Gangrenous comb
2	2067	0.25	5 min.	Moderate coloration
			30 min.	Pronounced coloration
			1 hr.	Excessive cyanosis
3	1815	0.20[a]	5 min.	Slight coloration
			30 min.	Moderate coloration
			1–1½ hr.	Characteristic cyanosis
4	1635	0.175	10 min.	Slight coloration
			30 min.	Moderate coloration
			1–1½ hr.	Characteristic cyanosis
5	1570	0.15	1 hr.	Partial coloration
6	1298	0.125	1 hr.	Slight coloration
Series B				
Series B				
2	2067	0.17	1–1½ hr.	Characteristic cyanosis
3	1815	0.165[b]	1–1½ hr.	Characteristic cyanosis
4	1635	0.16	1–1½ hr.	Slight to partial coloration
4	1635	0.17	1–1½ hr.	Characteristic cyanosis
2	2067	0.165	1–1½ hr.	Characteristic cyanosis
3	1815	0.16	1–1½ hr.	Slight to partial coloration
3	1815	0.17	1–1½ hr.	Characteristic cyanosis
4	1635	0.165	1–1½ hr.	Characteristic cyanosis
2	2067	0.16	1–1½ hr.	Slight to partial coloration

Threshold dose of cockerels (Series B) 0.5 cc./Kg.
Relative activity 250–300+.

$$\frac{\text{Threshold dose}}{\text{Dose unknown fluidextract}} \times 100 = \text{relative activity.}$$

[a] Maximum threshold.
[b] Minimum threshold.

REFERENCES

(1) Stuhr, E. T., THIS JOURNAL, 8 (1931), 794.

(2) "The Pharmacopœia of the United States of America," Eleventh decennial revision, Mack Printing Co., Easton, Pennsylvania, (1936), p. 148.

(3) Allport, N. L., and Jones, N. R., *Quart. J. Pharm. Pharmacol.*, 14 (1941), 106.

A Contribution to the Pharmacology of Xanthium*

By John C. Krantz, Jr., C. Jelleff Carr and Frederick K. Bell†

An Indian guide in the Maine woods called to the attention of one of our acquaintances, the use of a tea of Xanthium in the treatment of arthralgias. This information was communicated to us and during the last 3 years we have investigated the pharmacology and chemistry of this ubiquitous plant. Xanthium or cockle bur or spiny clotbur is a common weed which was naturalized in this country from Europe. The burs used in this investigation were identified pharmacognostically as *Xanthium spinosum* by Professor Heber W. Youngken, Chairman of the Subcommittee on Botany and Pharmacognosy of the United States Pharmacopœial Revision Committee.

* Received May 6, 1943, from the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, Md.

This investigation has been supported by the generosity of Mr. Charles McManus of Baltimore, Md.

† The authors are indebted to Dorothy Kibler, Anne Kunkel, Edward Merdinyan and Murray Finkelstein, formerly of this laboratory, for assistance in the prosecution of these studies.

EXPERIMENTAL

Preparation of Xanthium Extract.—The burs were air-dried and coarsely comminuted. The powder was extracted with boiling water repeatedly and the aqueous percolate was concentrated *in vacuo* to the consistency of a dry mass. This was powdered when cool. The extract was of a light brown color and possessed an odor resembling scorched chocolate. It was stable in the air and redissolved partially in water. In these studies extracts were prepared only from the dried burs. The yield is approximately 10%. Our experiments showed that the activity described in the following paragraphs was not present in the other organs of the plant.

Feeding Studies.—Four *Macacus rhesus* monkeys, 3 to 4 Kg., were fed 0.5 Gm. of Xanthium extract daily over a period of 3 months. Weekly determinations were made of body weight. The animals' weights remained constant within normal variations, and they did not exhibit diarrhea. Serum-calcium levels and blood-urea-nitrogen levels did not change significantly during the feeding period. Likewise, the blood-uric acid level remained significantly the same throughout the period of Xanthium ingestion. Bromsulfalein excretion tests modified for the monkey (1) were not affected by Xanthium feeding. At the termination of the experiment 4 animals were sacrificed and their kidneys and livers examined histopathologically by Dr. Henry Wollenweber of this department. There were no significant pathological findings in either the liver or kidneys.

Over a period of 2 yr. several hundred thousand 0.15-Gm. Xanthium extract tablets have been taken in clinical studies and no untoward effects have been observed.

Pharmacodynamic Activity.—Two per cent solutions of Xanthium extract in normal salt solution were administered intravenously to dogs (50 animals) under nembutal or ether anesthesia. The response elicited was a prompt fall in blood pressure with a rapid return to the normal level. Generally 0.5 to 1 cc. of the 2% solution reduced the arterial tension 30 mm. Animals acquired no tolerance to repeated injections. In one dog the twenty-third intravenous injection elicited a depressor response equal in magnitude to that of the first injection. As small a volume as 0.1 cc. of the 2% solution causes a 10-mm. fall in blood pressure. The response was not due to the injection of inert colloids as passage of the solution through a Berkefeld filter did not vitiate its depressor response. The fall in blood pressure was not accompanied by any marked change in respiratory rate or volume. The depressor response persisted after bilateral vagotomy. Nicotinization until vagal response was obliterated did not inhibit the characteristic depressor response of the Xanthium solution. The spinal dog likewise showed a fall in blood pressure upon the injection of Xanthium. The depressor response of the Xanthium solution was antagonized completely by the addition of an equal volume of epinephrine hydrochloride, 1:100,000 solution (Fig. 1).

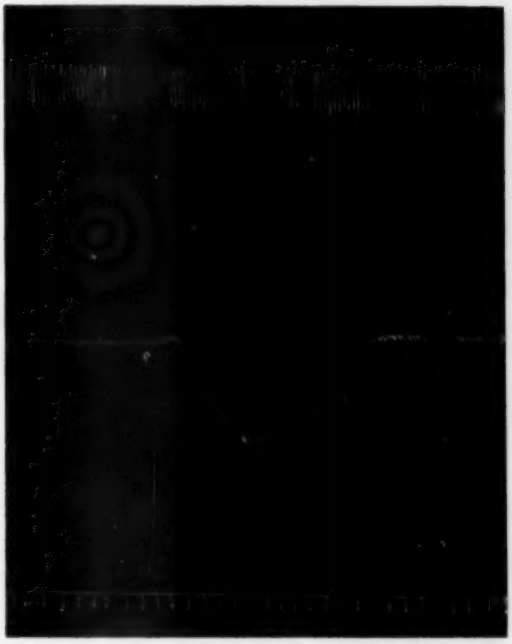

Fig. 1.—Effect on blood pressure.
Dog, 10 Kg.; under Ether Anesthesia intravenous injection of 3 cc., 2 % Xanthium Extract Solution

Atropine previously injected obliterated the Xanthium depressor response, which suggested the presence of choline or a choline ester. On the perfused frog's heart Xanthium extract (purified) produced a choline-like response which was antagonized by atropine. The Xanthium activity on the frog's heart was not obliterated by standing at room temperature for 10 min. with 2 *N* sodium hydroxide (2) indicating the presence of choline unesterified.

Isolation of Depressor Principle.—In a typical experiment, 400 Gm. of the crude aqueous extract of Xanthium burs was rendered fat-free by repeated extraction with petroleum ether. The dry residue was treated with 3000 cc. of warm water (50–55°) and thoroughly mixed. The mixture was shaken frequently, allowed to stand overnight and then treated with concentrated hydrochloric acid until no further precipitation was produced. Approximately 35 cc. of acid was added. The mixture was filtered. The residue on the filter, when dried, weighed approximately 270 Gm. and represented the undissolved portion of the original extract and the hydrochloric acid precipitate.

To the clear deeply colored filtrate was added quickly with stirring a boiling mixture of 1000 cc. of water and 50 Gm. of lead chloride which produced at once a dark yellow precipitate. This mixture was stirred frequently and allowed to

stand overnight. The supernatant liquid showed no precipitation on the further addition of lead chloride solution and the mixture was filtered. The precipitate, when dried, weighed approximately 110 Gm. and contained some undissolved lead chloride.

The clear filtrate, deep amber in color, was saturated with hydrogen sulfide and then filtered. The filtrate was treated with 50 Gm. of animal charcoal, thoroughly mixed and filtered at once. The clear filtrate was treated again with animal charcoal. After subsequent filtration a clear, straw-colored solution was obtained having a total volume of about 5 liters. This solution was concentrated at approximately 40° C. under reduced pressure to a volume of 1000 cc. Dry sodium bicarbonate was added in small quantities with constant stirring until the solution was just acid to litmus. The solution which became considerably darker in color but remained clear was concentrated to a syrupy consistency at 40° C. under reduced pressure and then treated with 1000 cc. of absolute alcohol.

The alcohol mixture was filtered, yielding a clear filtrate and a precipitate brown in color and of taffy-like consistency which became almost black on exposure to the air. No attempt was made to dry this precipitate completely. The weight was approximately 100 Gm. The filtrate was concentrated as before to a syrupy liquid, mixed with 500 cc. of absolute alcohol and filtered. The precipitate was mostly granular material with some syrupy admixture and weighed approximately 21 Gm. The procedure of concentrating the filtrate and subsequent addition of absolute alcohol was repeated until practically no precipitation occurred when the absolute alcohol was added and this stage was reached with the ninth treatment. The final filtrate was concentrated to a volume of approximately 50 cc. to which was added slowly and with constant stirring 100 cc. of a 30% solution of mercuric chloride in absolute alcohol.

A precipitate, definitely crystalline and light tan in color, began to form almost immediately and the mixture was filtered after standing a short time. The precipitate was treated with boiling acetone and the acetone-insoluble material was removed by filtration. Absolute alcohol was added to the clear water-white filtrate and the mixture was concentrated on the water bath until crystallization started. On cooling the mixture was filtered and the precipitate was washed with a little absolute alcohol and dried. The white crystalline material thus obtained, weighing approximately 3 Gm. and melting at 172–176°, was treated with 50 cc. of water, yielding a milky mixture with some undissolved material. On warming gently most of the material dissolved and the solution was slightly opalescent. The warm solution was saturated with hydrogen sulfide and

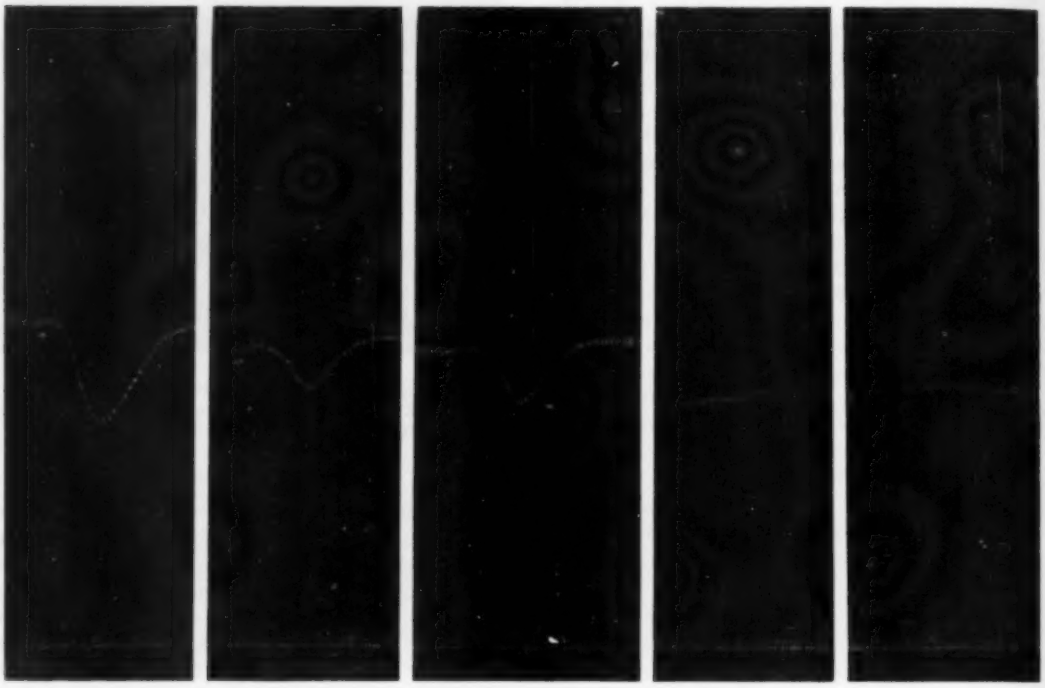

Fig. 2.—Blood pressure and respiration tracing of a dog under ether anesthesia.

Intravenous injections of No. 1. 2 cc. of Xanthium extract. No. 3. 5 mg. of choline chloride. No. 4. 5 mg. of purified Xanthium principle. No. 5. 5 mg. of choline chloride after atropinization. No. 7. 5 mg. of purified Xanthium principle.

0.5 Gm. of animal charcoal was added. The mixture was filtered at once and the filter was washed several times with small amounts of water. The combined filtrate and washings were evaporated almost to dryness under reduced pressure when the concentrate was transferred to a low-form wide-mouthed weighing bottle which was placed in a vacuum desiccator over calcium chloride and caustic potash.

Toward the end of the evaporation white crystals separated but several days' further treatment in the vacuum desiccator was necessary to remove the hydrochloric acid present. The final product weighed 1.1 Gm. It was readily soluble in water and very hygroscopic. The crystals were double refracting and gave the characteristic trimethylamine odor when treated with alkali. Small portions of a dilute aqueous solution of the crystals were examined under the microscope after the addition of selected reagents. Gold and platinum salts were readily formed in well-defined optically active crystals. Picric acid and Mayer's reagent also precipitated optically active crystals which were formed considerably more slowly than in the case of the platinum or gold salt. Both Dragendorf's and Wagner's reagents gave an immediate copious precipitate in the form of droplets.

For the identification of the product we have employed the method of Hofmann and Hobold (3) who prepared and studied the nitrate ester of choline perchlorate. By this procedure we obtained a well-defined and uniformly crystalline product, which, on recrystallization from acetone, melted at 187–188°. The derivative prepared in a similar manner from choline hydrochloride melted at 188–189°. A mixture of the two substances showed a melting range of 187–189°. However, remelting was not possible since some decomposition appears to take place at the melting point, a phenomenon which was also observed in taking the melting point of each of the constituents.

Microanalysis in duplicate yielded the following results as compared with the values calculated for the formula $(CH_3)_3N \cdot ClO_4 \cdot CH_2 \cdot CH_2 \cdot ONO_2$:

Calculated: C, 24.15%; H, 5.27%; N, 11.27%.
Found: C, 24.95%, 24.93%; H, 5.61%, 5.50%; N, 11.06%, 11.20%.

The chemical evidence indicates very strongly that the substance isolated was choline.

A comparison was made between the activity of the Xanthium extract and the isolated choline pharmacodynamically. Figure 2 shows a comparison between the activity of Xanthium extract and the active principle on the blood pressure of the dog.

DISCUSSION

Extracts of Xanthium frequently contain reducing sugars. As yet, the authors have not been able to isolate a glycoside from the plant. There are no alkaloids demonstrable in the plant. It is indeed questionable if the beneficial effects of this plant extract in arthralgias can be attributed to its choline content. Experiments on rats conducted according to the technic of Macht and Macht (4) failed to show that Xanthium produced analgesia. It is well established that certain choline derivatives such as β-methylacetylcholine chloride (Mecholyl) are useful in certain cases of arthralgias, perhaps through the mechanism of peripheral dilatation. It has been observed clinically that repeated doses of Xanthium extract possess hypotensive activity, which is marked in patients with hypertension.

In this laboratory we were unsuccessful in evaluating the efficacy of the extract against experimental arthritis produced by organisms of the pleuropneumonia group in mice as we found it impossible to produce the lesions reported by Sabin (5). In addition, a search for some other potent agent in the bur will be continued, which, if discovered, might further elucidate the beneficial results obtained in arthralgias by the administration of Xanthium extract.

CONCLUSIONS

1. An extract of Xanthium burs has been prepared and found innocuous when administered to *Macacus rhesus* monkeys.

2. The depressor response of the Xanthium extracts is attributable to choline which has been identified or some precursor of this amine.

3. The use of Xanthium in arthralgia has been discussed.

REFERENCES

(1) Krantz, J. C., Jr., Carr, C. J., Forman, S. E., and Evans, W. E., Jr., *J. Pharmacol.*, 69 (1940), 207.

(2) Gaddum, J. H., *Ann. Rev. Biochem.*, 4 (1935), 318.

(3) Hofmann, K. A., and Höbold, K., *Ber.*, 44 (1911), 1766.

(4) Macht, D. I., and Macht, M. B., THIS JOURNAL, 19 (1940), 193.

(5) Sabin, A. B., *Science*, 89 (1939), 228.

The Microscopical Identification of Quinacrine*

By Geo. L. Keenan

Quinacrine hydrochloride, now officially recognized in U. S. Pharmacopœia, XII, has assumed a more important role as an antimalarial because of the present scarcity of cinchona products. Quinacrine is also known by the names "Atabrine" and "Mepacrine." In connection with the accumulation of information descriptive of quinacrine, the occasion arose for a determination of the optical-crystallographic constants. From a microscopical examination of both the base and the salt, the following descriptive data were obtained and a technique developed for producing suitable crystalline material for microscopical identification from both tablets and powder.

In mass, both the base and the salt (dihydrochloride) are yellow, the former being somewhat lighter in color than the latter. The base consists of small yellow prismatic forms in ordinary light and these are best observed at a magnification of ×200. The prisms extinguish sharply with crossed nicols and show no interference figures with convergent polarized light (crossed nicols). The refractive indices, determined by the immersion method, are: $n_\alpha = 1.500$ (± 0.002) and $n_\gamma = < 1.733$. However, many fragments match the latter value.

The rhombic crystals of quinacrine dihydrochloride dihydrate extinguish sharply with crossed nicols and no interference figures are observed in convergent polarized light (crossed nicols). The refractive indices determined by the immersion method are: $n_\alpha = 1.522$ (± 0.002), $n_\gamma = < 1.733$, and many of the rhombs match the latter liquid. It is of interest that both the base and the salt give a brilliant red color when the undissolved material is warmed in a drop of Millon's reagent. This color does not appear in the cold.

In order to prepare quinacrine dihydrochloride dihydrate in a suitable form for microscopic study from either the powder or tablet material, the following technique has been developed. A small amount of quinacrine dihydrochloride powder (approximately 0.5 mg.) is dissolved in 2 drops of 5% ethyl alcohol on a regular 3 x 1-in. glass slide. A small fragment of potassium nitrite is then dissolved in the preparation. The formation of numerous thin yellow and frequently elongated rhombic plates may be hastened by slight warming on a hot plate. The addition of potassium nitrite serves aparently as a salting out medium and does not enter into the composition of the quinacrine hydrochloride dihydrate. This was confirmed by demonstrating that the optical crystallographic constants for the salt crystallized in this manner are identical with those obtained for the substance when normally crystallized with water. Rosenthaler (1) has also observed the formation of rhombs with sodium nitrite but offered no explanation as to the identity of the end product. Some foreign manufacturer's tablets were available for study and the microscopical examination of these tablets gave the same optical-crystallographic data as the domestic tablets.

SUMMARY

The optical-crystallographic properties of the quinacrine base and the quinacrine dihydrochloride dihydrate and a technique for crystallizing the salt in a form suitable for microscopical study have been described. A color reaction with both the base and salt and Millon's reagent has been observed.

REFERENCE

(1) Rosenthaler, L., *Pharm. Ztg.*, 78 (1933), 699.

* Received April 15, 1943, from the Microanalytical Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.